

## DIAGNOSIS OF VENEZUELAN EQUINE ENCEPHALOMYELITIS VIRUS BY IMMUNOFLUORESCENCE

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*Summary.* — The rapidity and sensitivity of Venezuelan equine encephalomyelitis (VEE) viral antigen demonstration by direct staining with fluorescein isothiocyanate- (FITC-) labelled rabbit immune globulin was checked. In HeLa cells the antigen was demonstrated as early as 12 hours after inoculation with massive doses, but after small doses ( $5 \times 10^3$  intracerebral -ic- mouse LD<sub>50</sub>) it was not detectable even after 44 hours. In chick embryo cells (CEC), antigen detection was linked with the appearance of a cytopathic effect. The susceptibility of CEC was higher than that of HeLa cells, but the finely granular structure of the fluorescent particles in CEC was difficult to evaluate. After inoculating test materials ic into suckling mice, VEE viral antigen was detectable as late as after 2–3 days.

To make diagnostic procedures more rapid, certain authors used haem-agglutination (HA) in demonstrating viral antigen in isolation experiments. Srihongse (1966) described the preparation of HA antigen from infected mouse brains and Gaidamovich and Vagzhanova (1965) proposed a HA method for use with cell cultures. The demonstration of VEE viral antigen in cell cultures by immunofluorescence was described by Metzger *et al.* (1961) and Hahon and Cooke (1967).

The VEE virus strain labelled "Venezuela", passed in mouse brains, was obtained in 1966 from the Institute of Virology, U.S.S.R. Academy of Medical Sciences, Moscow.

Cell cultures were grown on  $20 \times 8$  mm coverslips placed singly in tubes, which were each seeded with  $4 \times 10^5$  HeLa cells or  $2 \times 10^6$  CEC in 2 ml medium. The cultures were employed after 3 days of growth, when the cell counts per coverslip amounted to 251000 (HeLa) or 337000 (CEC). The coverslip cultures were overlaid with the given virus concentration in 0.2 ml medium. After 1 hour at 37° C in humid atmosphere the cultures were washed and incubated for various periods at 37° C in tubes with 2 ml fresh medium until examined.

The globulin was obtained from rabbits immunized with 4 doses of CEC-grown VEE virus. For the first dose, the virus was inactivated with formalin. The labelling of the globulin with FITC was carried out by F. Škvařil, Research Institute of Immunology, Prague. The serum used for the preparation of conjugate had a neutralization antibody titre of 1 : 20240 as determined in suspension CEC cultures with 100–500 TCD<sub>50</sub>/0.1 ml of virus. The conjugate contained 1.56% proteins; the FITC: protein ratio was 10.57, the staining titre 1 : 32 (working dilution 1 : 8).

The preparations were contrast-stained with rhodamine (Benda *et al.*, 1968).

To determine the time needed for making possible the visualization of VEE viral antigen in infected HeLa cell cultures, we carried out the following experiment: Two groups of coverslip cultures were infected with doses of

$5 \times 10^7$  and  $5 \times 10^5$  ic mouse LD<sub>50</sub> of VEE virus, respectively. Coverslips were taken out at intervals and treated with the conjugate. The results (Table 1) indicate that after a sufficiently large inoculum, VEE viral antigen

Table 1. Experiments on rapid detection of VEE viral antigen in HeLa cells

Hours after inoculation	Findings in cultures infected with	
	$5 \times 10^7$ LD <sub>50</sub>	$5 \times 10^5$ LD <sub>50</sub>
9.5	Negative	Negative
12	Fluorescent cytoplasm in single or occasionally two adjacent cells	Negative
15	Fluorescent cytoplasm in single cells or in foci of up to 10 cells (Figs 1 and 2)	Negative
20	Fluorescent cytoplasm of cells in numerous, even confluent foci consisting of up to 50 cells	Fluorescent cytoplasm of a few single cells
24	Ditto	Fluorescent cytoplasm of single cells or of widely scattered foci of up to maximally 7 cells

Uninfected control cultures were negative at all intervals tested.

could be demonstrated by immunofluorescence as early as 12 hours p.i. With the smaller inoculum, antigen detection was delayed till the 20th hour p.i. With an inoculum of approximately  $5 \times 10^3$  ic mouse LD<sub>50</sub> the inoculated HeLa cell cultures showed no fluorescence even after 44 hours.

In a second experiment we tested the possibility of using CEC cultures for immunofluorescence assay of VEE viral antigen. Groups of coverslip CEC cultures were inoculated with mouse brain suspension of VEE virus, the doses being  $5 \times 10^7$ ,  $5 \times 10^5$  and  $5 \times 10^3$  ic mouse LD<sub>50</sub>, respectively. After adsorption and washing, the cultures were incubated for 8, 10, 12, 15, 20, 24 and 38 hours. Thereafter they were fixed with acetone and treated with the conjugate.

The results are summarized in Table 2. As compared to HeLa cells, the onset of fluorescence was somewhat delayed even after the greatest dose, but CEC proved to be more satisfactory for the demonstration of antigen after small inocula. As distinct from HeLa cells, the visualization of antigen in CEC was linked with the time of appearance of the microscopic cytopathic effect, namely rounding and disintegration of cells. At low magnification, as distinct from HeLa cells, the fluorescence was not easily discernible. In general, the fluorescence was shown by fine granules which filled at the onset the narrow cytoplasm of rounded cells or the cytoplasm of not yet rounded large bar-shaped cells or their outgrowths and later cell fragments or remnants of filamentous outgrowth which did not retract after the cells had disintegrated.

The diagnostic value of immunofluorescence assay with impression smears from mouse brains was tested in two isolation experiments. Suckling mice were ic inoculated with blood from 2 persons showing for 1—2 days a febrile illness, suspected as laboratory VEE infection. Mice killed 2 days and dead

Table 2. Experiments on rapid detection of VEE viral antigen in CEC

Hours after infection	Findings in cultures infected with		
	$5 \times 10^7$ LD <sub>50</sub>	$5 \times 10^5$ LD <sub>50</sub>	$5 \times 10^3$ LD <sub>50</sub>
8	Negative	Negative	Negative
10	Negative	Negative	Negative
12	Negative	Negative	Negative
15	Numerous rounded cells with a narrow fluorescent zone of cytoplasm. Granular structure	Single rounded cells showing fluorescence. Granules in cell outgrowths	Negative
20	Ditto	Ditto	Fluorescence of single rounded cells
24	Appearance of fine, fluorescent cell debris	Fluorescence of numerous cells and cell debris	Ditto
38	Fluorescence of cell debris and of granules in original outgrowths	Ditto	Fluorescence of numerous cells and cell debris

In uninfected control cultures there occurred only sporadic nonspecific and little marked fluorescence of some undeveloped cells.

3 days after inoculation were dissected and impression smears prepared from their brains. Fluorescence of single or foci of nerve cells was seen in the smears. Subsequent serological diagnosis confirmed the isolation of VEE virus.

#### Discussion

The sensitivity of HeLa cells to the effect of VEE virus is comparatively low as are the titres reached in these cells by the mouse brain-adapted strain of VEE virus. We assume that this fact is also reflected by the low sensitivity of immunofluorescence assay of VEE virus antigen in HeLa cells. At the same time we think that, due this limited sensitivity of HeLa cells, voluminous and clearly fluorescent structures of antigenic material may accumulate in non-disintegrated cells. Therefore we consider HeLa cells as suitable for the demonstration of viral antigen after inoculation of large doses of virus and for estimation of the efficiency of the conjugate, but not for the isolation of VEE virus from the blood.

The shortest period after inoculation of HeLa cells with large inoculum for the appearance of fluorescent antigen (12 hours) was similar to that reported by Metzger *et al.* (1961). These authors observed fluorescence 10 hours

after inoculation of a guinea pig heart cell line with a large, not definitely determined, dose of virus. They worked, however, with a virus possessing lowered virulence for laboratory animals and adapted by 80 passages to the given cells. Hahon and Cooke (1967) observed the onset of fluorescence in McCoy's synovial human cells as early as at 8 hours, when using the centrifugation technique to enhance adsorption of the Trinidad strain onto the cells.

CEC proved to be relatively sensitive both to the cytopathic effect of virus and for immunofluorescence assay of viral antigen. But the rapid cytopathic changes result in the formation of a finely granular, considerably dispersed antigen whose fluorescence is more difficult to evaluate and especially to photograph. Our limited experience suggests that a clearer picture could be obtained with impression smears in isolation experiments in mice.

The results of our investigations (Hrušková *et al.*, 1969; and to be published) and those by Kundin *et al.* (1966) and Kundin (1966) indicate that direct demonstration of VEE viral antigen in organs from infected animals is difficult, it being rarely positive or little distinct. Because the organs from infected animals contain, in general, high levels of virus, it is possible to conclude that for rapid assay of viral antigen it might be appropriate to inoculate organ suspensions into HeLa cell cultures and to examine the latter by immunofluorescence.

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#### Explanation of Photomicrographs:

HeLa cells infected with VEE virus (see Table 1).

*Fig. 1.* Fluorescence of a cell focus. Approx.  $\times 250$ .

*Fig. 2.* Fluorescent antigen in the cytoplasm showing finely granular or lumpy till confluent structure. Approx.  $\times 650$ .